Lactobacillus adhesion to epithelial cells from bovine vagina

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Bacterial adhesion to epithelial cells is one of the characteristics available to support the capability of microorganisms to colonize the specific tract of the host. This property is studied both in pathogens and probiotic bacteria. The aim of this work was to evaluate and characterize the adhesion to vaginal bovine epithelial cells of three bovine probiotic *Lactobacillus* strains with different surface properties. An in vitro assay was set up and optimised by incubation of the microorganisms with fresh vaginal epithelial cells. The evaluation of the bacteria adhered was observed by sacanning electron microscopy and quantified through Gram stains. Previous treatments with proteolytic enzymes and sodium periodate were applied to determine the chemical nature of the responsible factors. The adhesion patterns were different for each one of the strain and coincident with the autoaggregative phenotype of strains that showed also to be the more adherent. The factors associated with adhesion to vaginal epithelial cells in the three strains are glycoproteins in nature. The projection of the results in a product for veterinarian proposes is discussed.

Keywords: adhesion, Lactobacillus, epithelial cells, probiotic, bovine vagina

1. Introduction

The adhesion to epithelial surfaces is a critical step in the colonization of *Lactobacillus* and one of the suggested mechanisms by which they could protect the vagina of the colonization of pathogens, by their competition for the receptor sites for adhesion [1, 2]. Consequently, the capability of adhesion to epithelial cells has been suggested as one of the main characteristics to be used for the selection of probiotic strains [3]. The bacterial adhesion to mucosal surfaces includes interactions that take place between specific adhesins of the bacterial surface and receptors of host tissues [4]. The pathogenic microorganisms that have their way of entrance through the mucous needs to adhere to the surface of the epithelial cells as the first event of the next infection, as for example in *Trichomonas foetus* infection, that produces infertility by chronic metritis in bovine females. This parasite adheres better and in greater degree to queratinized than to non-queratinized epithelial cells. This union is specific because the first contact is produced by the posterir area of the flagellum, but later, by the entire cell [5].

Pathogenic microorganisms and those that are part of the normal microbiota have shown to posses macro-molecules in their surface that participate in their adhesion to epithelial cells. In the genus *Lactobacillus*, numerous adhesins have been characterized, mainly those responsable for the union to the cells of intestinal epithelium. The available literature shows a very wide variety of hosts, from humans to different animal species [6,7]. More recently, those adhesins that participate in the adhesion to urogenital epitelium [8] have been studied.

The adhesion is one of the strategies used by many bacteria to maintain stable numbers at different ecological niches. Some studies have shown that the a high capability of adhesion of a certain microorganism indicates that they can compete for nutrients with a higher efficiency than those nonadherent bacteria [9].

The methods applied to “in vitro” determination of bacterial adhesion are performed by the incubation of epithelial cells obtained from the specific host with those bacteria of interest, at certain conditions. The microorganisms can be labelled either with radioactive or fluorescent substrates. Then, nonadhered bacteria need to be separated by differential centrifugation or filtration, and finally, the adhered bacteria quantified by the degree of radioactivity, fluorescent content, or stained with different stains. The most frequently used method for the evaluation of the adhesive properties of *Lactobacillus* to epithelial cells was described by Mardh and Weström [10]. According to this method, the number of bacteria adhered to the surface of the epithelial cells is determined through their quantification by microscopic observation. Many scientist have published modifications of this method at different degree [8, 11, 12, 13]. In the present paper, the method originally described by Mardh and Weström [10] with slight modifications, was applied to study the adhesion of bovine vaginal lactobacilli to epithelial cells scrapped

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from bovine vagina. It was performed to evaluate the capability of adhesion of selected probiotic *Lactobacillus* strains to bovine vaginal epithelial cells. The microorganisms studied showed beneficial properties, as production of inhibitory substances [14, 15] and also some technological characteristics [16] which suggest they could be good probiotic candidates to prevent infectious metritis in the bovine female tract.

2. Materials and Methods

2.1 Microorganisms and culture conditions

The microorganisms used were previously selected by their beneficial characteristics: hydrophobic surface, self-aggregating patterns and capability to produce antimicrobial substances [14, 15, 16]. They were: *Lactobacillus gasseri* CRL (Centro de Referencia para Lactobacilos Culture Collection)1412, *Lactobacillus gasseri* CRL 1421 and *Lactobacillus delbrueckii sp. delbrueckii* CRL 1461, identified by genetic methodologies [14]. They were stored in LEL (10% low fat milk, 0.5% yeast extract, 1% glucose) at -20ºC, and subcultured three times during 12-14 hours at 37° in LAPTg broth (1.5% peptone, 1% tryptone, 1% glucose, 1% yeast extract, 0.1% Tween 80, pH 6.5), before the adhesion assay.

2.2.Vaginal epithelial cells

The cells were collected by scrapping the vaginal wall of healthy cows with a cytobrush (Cyto Soft Brush, Medical Packaging Corp.,USA), and resuspended immediately in MEM (Eagle´s Minimal essential media, Gibco) pH 7. They were refrigerated until the adhesion assay.

2.3. Preparation of the microorganisms and cells suspensions

Previous to the adhesion assay, the bacterial and cells suspensions were standarized as follows: *Lactobacillus* harvested from a 12h culture, were washed twice with saline solution (0.8% NaCl) and once with MEM pH 7, and later resuspended in MEM to obtain a final concentration of 10^7 CFU/ml (OD_{540}=0.15). Alternatively, acridine orange can be used to stain microorganisms when adhesion is going to be evaluated by fluorescence microscopy: bacterial pellet was resuspended in equal volume of 0.01% acridine orange solution, incubated for 10 minutes and washed with MEM until complete elimination of the stain from the supernatant.

The indigenous bacteria were removed by washing three times with 10 ml of MEM and centrifugating at 800rpm at 2°C during 10 minutes before the adhesion assay. The complete elimination of these microorganisms was controlled by microscopic observations. Vaginal cells suspensions were adjusted to 10^5 cells/ml as determined in Neubauer cytometric chamber under light microscope.

2.4. Adhesion assay

500ul of bacterial suspension was added to 500ul of vaginal cell suspension and later incubated at 37°C for 1 hour in agitated conditios (low agitation 35 opm). The control was prepared by substituting the bacterial suspension with MEM. In order to remove non-adherent bacteria, the tubes were centrifuged for 10 minutes at 800 rpm, the supernatant discarded, the pellet resuspended in 1ml MEM, and washed three time under the same conditions. In this pellet, the bacterial binding to the epithelial cells was examined by electron microscopy and light microscopy (Giemsa staining). The total number of cells, cells with associated bacteria and bacteria adhered to the cell were quantified. The results were expressed as:

a) Percentage of adhesion: the number of epithelial cells with bacteria adhered was divided by the total number of epithelial cells observed x 100.

b) Adhesion index: the total number of bacteria attached to cells was divided by the number of cells with bacteria adhered.

The application of the two index allowed us to evaluate not only the efficiency of adhesion, but also the differnent patterns of adhesion.

2.5. Evaluation of adhesion by electronic microscopy

The scanning electron microscopy was applied to demonstrate the adhesion of lactobacilli to bovine vaginal epithelial cells and to evaluate the patterns of adhesion, according to the different phenotypes of bacterial aggregation. The adhesion tests were performed as described previously. Once removed the nonadhered bacteria, the pellets were fixed with Laptg/glutaraldehyde solution (3% glutaraldehyde in 0.1M pH 7.4 phosphate buffer). They were incubated during 30 minutes at 25ºC, and later centrifuged during 10 minutes at 2000 r.p.m, and the pellets resusspended with 3% glutaraldehyde. The post-fixation step was performed with OsO4 (1% in the same buffer). Finally, the samples were dehydrated with increasing acetone-alcohol concentrations, dried in a critical-point dryer, and mounted coated with gold. The specimens were examined in a Joel JSM35CF scanning electron microscope.

2.6. Chemical nature of adhesion factors

In order to evaluate the chemical nature of the bacterial factors involved in the adhesion, the assays were performed in the conditions described previously, but using bacteria previously treated with: 50mM sodium periodate (in acetate buffer pH 4.5), 2mg/ml Lipase (in acetate buffer pH 5), 2.5 mg/ml trypsin (in phosphate buffer pH 7), 1mg/ml K proteinase (in PBS pH 7). The controls included the buffer solutions of each assay. Additional tests were performed with microorganisms washed with 2% SDS, to dissolve the more superficial bacterial protein layers without producing cells injuries. The results were then evaluated by counting the cells and bacteria by Gram stains.

3. Results

3.1. Adhesion capacity of bovine vaginal lactobacilli

The three strains of lactobacilli studied were able to adhere to bovine vaginal epithelial cells. There were no differences between their percentages of adhesion at pH 4.5 and all of them showed the same pattern of adhesion homogenously adhered to almost all the epithelial cells. However, the adhesion indexes were significantly differents, being *L. delbrueckii* subsp. *delbrueckii* CRL1461 the one showing the higher capability of adhesion, followed by *L. gasseri* CRL1421. *L. gasseri* CRL1412 showed the smaller adhesion index, being the most sensitive to the pH modifications from 4.5 to 7, since decreasements of both parameters were observed: this means that the number of bacteria adhered to each cell was affected and also the number of cells to which the bacteria were adhered.

The pattern of *L. gasseri* CRL1421 was different, being affected only the index of adhesion when pH values were modified. *L. delbrueckii* subsp. *delbrueckii* CRL1461 showed a different behaviour, because the modifications of pH did not induce significant alterations in any of the parameters evaluated (table 1). Different patterns of adhesion were also observed in strains with different aggregation phenotypes, in fresh preparations by fluorescent microscopy. *L. gasseri* CRL1412 showed an uniform distribution on the cellular surface, whereas *L. delbrueckii* subsp. *delbrueckii* CRL1461 adhered as clusters or bacterial aggregates (Figure 1).

Table 1. Adhesion indexes of three strains of bovine vaginal lactobacillli to epithelial cells at different pH.

	pH 4.5		pH 7	
	% of adhesion	Adhesion indice	% of adhesion	Adhesion indice
L. gasseri CRL1412	94.7±4.1	22.4±5	61.1±9.2	10.3±3
L. gasseri CRL1421	100±0	43.9±4.5	100±0	22.5±3.5
L. delbrueckii CRL1461	91.7±3	67.5±8.2	93.7±6.2	66.7±6.8

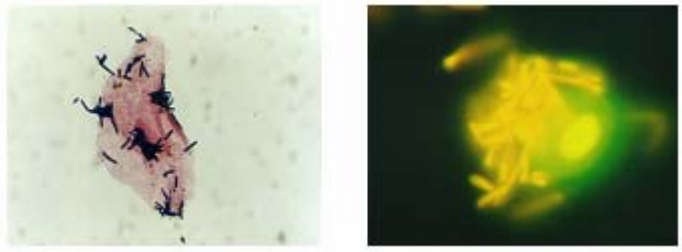

Figure 1. Gram and fluorescence stains showing the adhesion of *L. delbrueckii* sp. *delbrueckii* CRL 1461 at pH 7. This strains shows an autoagreggative pattern and adheres as clusters on the surface on the cells

3.2. Evaluation of the adhesion patterns by scanning electron microscopy

The microphotographies obtained from scanning microscopy illustrate the adhesion of *L. gasseri* CRL1412 and *L. delbrueckii subsp. delbrueckii* CRL1461 on the surface of bovine epithelial cells (Figures 2 and 3), without producing morphologic or ultrastructural modifications of the vaginal epithelial cells referred to the adhesion of lactobacilli. The scanning electron microscopy shows *L. gasseri* CRL1412 aggregated on the mucus and also *Lactobacillus* adhered to the surface of epithelial cells with or wihout mucus (Figure 2). Bacterial aggregates of *L. delbrueckii* subsp. *delbrueckii* CRL1461 are observed also on the surfaces of the cells (Figure 3) and covering groups of epithelial cells, although they were not aggregated with mucus fibers.

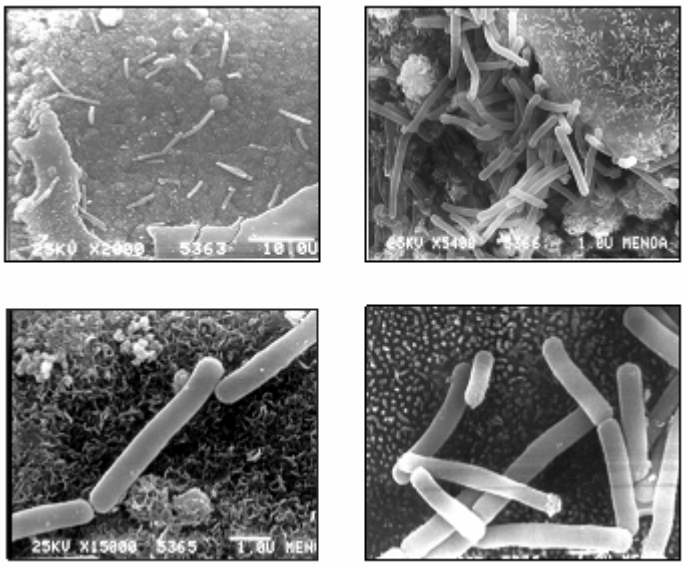

Figure 2. Adhesion of *L. gasseri* CRL1412 to epithelial cells of bovine vagina by scanning electron microscopy. a) Bacilli adhered to the surface of epithelial cells (2000x); b) aggregation of bacilli in presence of mucus adhered to the surface of a group of cells (5400x); c) adhered *Lactobacillus* to the cell surface covered (15000x) and d) free of mucus (15000x).

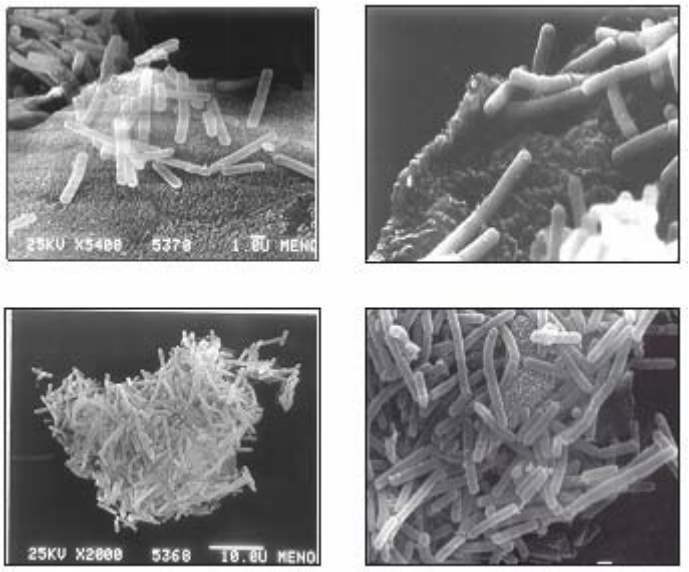

Figure 3. Adhesion of *L. delbrueckii subsp. delbrueckii* CRL 1461 to epithelial cells of bovine vagina by scanning electron microscopy. Bacterial aggregates on the surface of a group of epithelial cells: a) 5400x and b) 7800x. A group of epithelial cells covered with bacterial aggregates: c) 2000x and d) 5400x

3.3. Chemical nature of the adhesion factors

The results of chemical and enzymatic treatments help to determine the chemical nature of the bacterial structures involved in the adhesion of *L. gasseri* CRL1412 showed in Table 2. The high sensitivity of both indexes was observed when the bacteria were treated with periodate and lipase, which indicates that lipidic and carbohydrate factors could be involved in the adhesion. An increasement of the index of adhesion was observed with trypsin and K proteinase treatments, in addition the percentage of adhesion was higher when bacteria were treated with K proteinase (figure 4).

Table 2. Effect of different treatments on the adhesion of *L. gasseri* CRL1412 to bovine epithelial cells.

	% of adhesion	Adhesion index
Control[a]	94.7±4.1	22.4±5
Na Periodate	21.0±5.6	1.5±1
Control[b]	61.1±9.2	10.3±3
lipase	15±3.4	1.2±1
trypsin	72.7±5.4	25±4
K proteinase	100±0	34.9±3.5

[a]acetate buffer pH 4.5 Control.
[b] PBS pH 7 Control (for lipasae the pH was adjusted to 7.7)

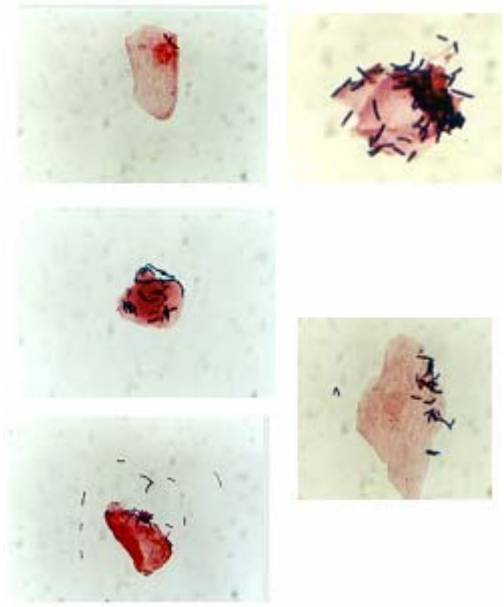

Figure 4. Effect of Sodium periodate (a), trypsin (c) and K proteinase (d) on the adhesion of *L. gasseri* CRL1412, and their respective control b) and e).

Similar behavior was found in *L. gasseri* CRL1421, where the percentage and index of adhesion decreased significantly by the treatment with sodium periodate (table 3), that suggests the participation of glucidic components in the adhesion factors. The treatment with trypsin increased the adhesion index, in a similar way than in *L. gasseri* CRL1412. The results are showed in figure 5.

Table 3. Effect of differents treatments on the adhesion of *L. gasseri* CRL1421 to epithelial cells.

	% adhesion	Adhesion index
Control[a]	100±0	43.9±4.5
Na Periodate	60.0±3.8	11.0±4.3
Control[b]	100±0	22.5±3.5
lipase	-	-
trypsin	100±0	33.8±3
K proteinase	95±3.9	24.4±4.6

[a]: Acetate buffer Control, pH 4.5. [b] PBS pH 7 control.

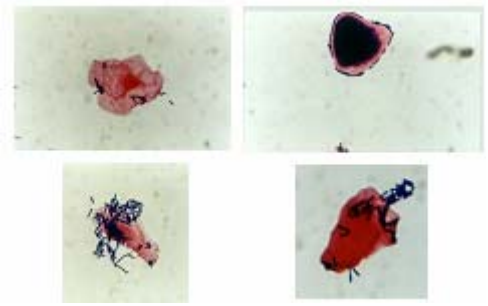

Figure 5. Effect of periodate and trypsin treatments on the adhesion *of L. gasseri* CRL1421. The upper images are the controls.

The effects of different treatments on the adhesion of *L. delbrueckii* subsp. *delbrueckii* CRL1461 to bovine vaginal epithelial cells are resumed in table 4. The adhesion parameters were modified in a very particular way, different from *L. gasseri* CRL1412 and *L. gasseri* CRL1421 behaviour. The treatment with sodium periodate affected the adhesion efficiency, producing a decresement of both parameters, which means that lower number of cells were able to adhere to the bacteria and also a lower number of bacteria were adhered per cells. Trypsin and K proteinase did not produced significant modifications of the percentages of adhesion, but the indexes of adhesion decreased considerably, in particular by trypsin. These results would indicate the glicoproteic nature of the factors involved in the adhesion phenomenon. In addition, a loose of the agreggative phenotype was observed (figure 6), which suggests that some of the sensitive structures would participate in the bacterial aggregation.

Table 4. Effect of different treatments on the adhesion of *L. delbrueckii subsp. delbrueckii* CRL 1461 from bovine epithelial cells.

	% of adhesion	Adhesion index
Control[a]	91.7±3	67.5±8.2
Na Periodate	66.7±2.5	9.8±5.8
Control[b]	93.7±6.2	66.7±6.8
Lipase	nd	nd
Trypsin	75.6±5	9.1±5.2
K proteinase	81.3±4.5	29.6±6

[a] Buffer acetate pH 4.5 control.
[b] PBS pH 7 control (for lipase the pH was adjusted to 7.7).

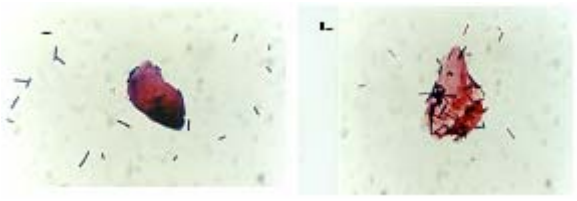

Figure 6. Effect of trypsin on the adhesion of *L. delbrueckii* subsp. *delbrueckii* CRL1461. (a) treated and (b) PBS control.

The persistence of the efficiency of adhesion after the treatment with 2% SDS in *L. gasseri* CRL1421 and *L. delbrueckii* subsp. *delbrueckii* CRL1461 suggests the existence of complex structures in the

membrane anchoring the adhesion components. After washing *L. gasseri* CRL1412 cells with 2% SDS, the parameters of adhesion decreased significantly (table 5), which would indicate that surface proteins, as S layer could be participating in this phenomenon, as seen in Figure 7.

Table 5. Effect of the treatment of 2% SDS on the adhesion of bovine vaginal lactobacilli to bovine epithelial cells.

	Control (saline solution)		SDS 2%	
	% of adhesion	Adhesion index	% of adhesion	Adhesion index
L. gasseri CRL1412	89.5±4.1	20.2±4.2	54.5±5.6	1.8±1
L. gasseri CRL1421	95.3±4.7	44±9.4	93.8±5	27.1±8
L. delbrueckii CRL1461	100±0	54.3±8	100±0	41.2±6

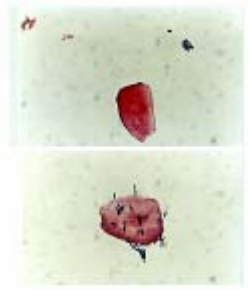

Figure 7. Adhesion of *L. gasseri* CRL1412 treated with 2% SDS a) treated b) control.

4. Discussion

The capability to produce antagonistic substances, the presence of structures related with adhesion to host tissues and the colonization to different mucosal sites promote the resistance of the indigenous microbiota to be displaced by many internal or external forces or factors. In the same sense, probiotic microorganisms must adhere to the host surfaces or mucosa to be able to colonize and exert their beneficial effect [17]. Then, at least two conditions must be fulfilled: the heterologous bacteria must be able to multiply and to remain at stable levels in situ. Basically, it depends on the bacterial capability to adhere to the epithelial cell and also to the mucus to avoid the expulsion [3]. In this sense, the capability of lactobacilli to adhere to the vaginal epithelial cells is an important step for the subsequent colonization and formation of a barrier on the mucosa, to prevent the income of pathogenic microorganisms [18]. Therefore, adhesion is one of the desired property for the adecquate selection of probiotic microorganisms [19, 20, 21 ,3].

In the urogenital tract, the probiotic *Lactobacillus* can adhere to inhibit the adhesion of pathogenic microorganisms, either by steric impediment or specific competition by adhesion sites [21]. In this work, the adhesion of three probiotic strains originally isolated from the bovine vaginal tract: *L. gasseri* CRL1421, *L. delbrueckii* subsp. *delbrueckii* CRL1461 and *L. gasseri* CRL1412 to bovine epithelial cells was demonstrated. The application of two parameters: percentage of adhesion and adhesion index allow to recognize different patterns of adhesion according to the aggregation phenotypes. The data obtained were used to evaluate the efficiency of adhesion and the chemical nature of the bacterial factors involved in the phenomenom.

The values of the parameters of adhesion obtained for the three vaginal lactobacilli studied demonstrated their high efficacy of adhesion to bovine epithelial cells. This specificity of the bacteria to the homologous tissues or ecological niche where they were originally isolated was also reported for human vaginal Lactobacilli [13]. The results presented in this paper also provides the basis to sustain the isolation and selection of homologous strains from the ecological niche where the probiotic product will be applied, host-specificity also demonstrated by other researchers in different epithelia [22, 23, 24].

The adhesion of *L. gasseri* CRL1412 and *L. gasseri* CRL1421 was sensitive to the modifications of pH, showing to be higher at pH 4.5 than at pH 7. Different behaviour was reported in human vaginal *Lactobacillus*, that did not show differences in their adhesion at pH 4 or 7 [13]. The results obtained with the two strains of *L. gasseri* suggest the glicosidic nature of the factors involved in adhesion to bovine

vaginal epithelial cells. In the experiments with *L. gasseri* CRL1412, the factors could be associate also to lipidic components. Tannock et al [25] suggested that the lipoteichoic acid participates in the adhesion of some lactobacilli to the scamous epithelial cells of the stomach [25]. More recents studies have associated the external proteic S-layer to the adhesion events [26].

A particular behavior was observed when bacteria were treated with proteolytic enzymes that increased the degree of adhesion, probably by generation of residues with a higher affinity to the eucariotic cell receptor. Similar behavior was described by Greene and Klaenhammer [27] for *L. gasseri* ADH, that duplicated the adhesion to Caco-2 human cells after the treatment with pepsin. Morata de Ambrosini et al [28] obtained similar results for *L. casei* CRL431 after the treatment with protease. The sensitivity of *L. delbrueckii* subsp. *delbrueckii* CRL1461 to enzymatic and chemical treatments reveals both, proteic and glycosidic nature (or glycoproteic) of the factors associated to adhesion. Probably these factors are also involved in the autoagreggation phenomenom, since the decrease of adhesion was coincident with a modification from agreggating to non-aggregating phenotype. Boris et al. [8] reported that the adhesion of *L. acidophilus* and *L. gasseri* to human vaginal epithelial cells is mediated by a glycoproteic receptor. The components of the bacterial surface responsible of the adhesion to the vaginal epithelial cells seem to be strongly linked to the structure of the cell wall in *L. gasseri* CRL1421 and *L. delbrueckii* subsp. *delbrueckii* CRL1461, since the treatment with 2% SDS did not affect significantly the adhesion parameters. Different results were obtained with *L gasseri* CRL1412 because the adhesion was lost completely after the treatment with SDS. Probably, the responsible structures for the adhesion could be related to the S layer consituents. The determinants responsible for the adhesion of lactobacilli to epithelial cells are of different chemical nature, from proteins [8, 30] S-layer constituents or lipoteichoic acid [18] to lectines or carbohydrates [27]. On the other hand, why lactobacilli remain adhered to the epithelial cells after the long and hard treatments and manipulations applied during the preparation of the samples for scanning electron microscopy, would indicate that strong forces of interaction between the bacteria and the cells are present to avoid their separation.

Previous results [14, 15, 16] and those obtained in this work allow us suggest the inclusion of *L. gasseri* CRL1421, *L. gasseri* CRL1412 and *L. delbrueckii* subsp. *delbrueckii* CRL1461 in a probiotic product to be applied in the bovine vaginal tract to prevent metritis.

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